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VACCINES AND ANTIVIRAL AGENTS

Alternative Virus-Like Particle-Associated Prefusion F Proteins as Maternal Vaccines for Respiratory Syncytial Virus

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ABSTRACT Maternal vaccination may be the most effective and safest approach to the protection of infants from respiratory syncytial virus (RSV) infection, a severe acute lower respiratory tract disease in infants and young children worldwide. We previously compared five different virus-like particle (VLP)-associated, mutation-stabilized prefusion F (pre-F) proteins, including the prototype DS-Cav1 F VLPs. We showed that alternative versions of prefusion F proteins have different conformations and induce different populations of anti-F protein antibodies. Two of these alternative pre-F VLPs, the UC-2 F and UC-3 F VLPs, stimulated in mice higher titers of neutralizing antibodies than DS-Cav1 F VLPs (M. L. Cullen, R. M. Schmidt, M. G. Torres, A. A. Capoferri, et al., Vaccines 7:21–41, 2019, https://doi.org/10.3390/vaccines7010021). Here we describe a comparison of these two pre-F VLPs with DS-Cav1 F VLPs as maternal vaccines in cotton rats and report that UC-3 F VLPs significantly increased the neutralizing antibody (NAb) titers in pregnant dams compared to DS-Cav1 F VLPs. The neutralizing antibody titers in the sera of the offspring of the dams immunized with UC-3 F VLPs were significantly higher than those in the sera of the offspring of dams immunized with DS-Cav1 VLPs. This increase in serum NAb titers translated to a 6- to 40-fold lower virus titer in the lungs of the RSV-challenged offspring of dams immunized with UC-3 F VLPs than in the lungs of the RSV-challenged offspring of dams immunized with DS-Cav1 F VLPs. Importantly, the offspring of UC-3 F VLP-immunized dams showed significant protection from lung pathology and from induction of inflammatory lung cytokine mRNA expression after RSV challenge. Immunization with UC-3 F VLPs also induced durable levels of high-titer neutralizing antibodies in dams.

IMPORTANCE Respiratory syncytial virus (RSV) is a significant human pathogen severely impacting neonates and young children, but no vaccine exists to protect this vulnerable population. Furthermore, direct vaccination of neonates is likely ineffective due to the immaturity of their immune system, and neonate immunization is potentially unsafe. Maternal vaccination may be the best and safest approach to the protection of neonates through the passive transfer of maternal neutralizing antibodies *in utero* to the fetus after maternal immunization. Here we report that immunization of pregnant cotton rats, a surrogate model for human maternal immunization, with novel RSV virus-like particle (VLP) vaccine candidates containing stabilized prefusion RSV F proteins provides significant levels of protection of the offspring of immunized dams from RSV challenge. We also found that antibodies induced by VLPs containing different versions of the prefusion F protein varied by 40-fold in the extent of protection provided to the offspring of vaccinated dams upon RSV challenge.

KEYWORDS maternal immunization, glycoproteins, immune response, respiratory syncytial virus, virus-like particles

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Respiratory syncytial virus (RSV) is a significant human pathogen resulting in particularly serious infections in infants and young children worldwide (1). The virus is a common cause of severe acute lower respiratory tract disease in this population, frequently resulting in hospitalization in the United States and significant mortality in developing countries (1–3). However, despite decades of effort, no licensed vaccines exist.

Attempts to develop RSV vaccines have been ongoing since the 1960s. The failure to identify an effective vaccine has been due in part to a lack of recognition of the role of the RSV F protein conformation in vaccine candidates for the stimulation of protective neutralizing antibody (NAb) responses. Like many viral fusion proteins, the RSV F protein is folded into a metastable, prefusion conformation which, upon activation, refolds into a structurally different postfusion conformation (4–8). The prefusion form of the RSV F protein (the pre-F protein) is by far the most effective form for inducing NAbs, but this form is quite unstable (7, 9). Thus, most vaccine candidates, until recently, were formulated with the postfusion form of the F protein (the post-F protein) (10).

Another problem with vaccine development has been the unique and significant concerns over the safety of the vaccine candidates. This issue arose from the failure of formalin-treated virus (FI-RSV), which not only was ineffective in preventing disease but also resulted in life-threatening enhanced respiratory disease (ERD) upon natural exposure to RSV (11–14). Thus, all candidates going forward must meet stringent safety standards.

Finally, most preclinical tests of RSV vaccines have been performed in naive animals, and preexisting immunity to RSV is often disregarded. Many of these prototype vaccines induced sterilizing immunity in experimental animals but then failed in clinical trials, where preexisting immunity to RSV may play an important confounding role.

Given the safety issues, considerations of the F protein conformation, and the immunological immaturity of infants combined with the inhibitory effect of maternal antibodies (matAbs), a current view is that maternal vaccination with vaccines containing the stabilized prefusion form of F protein may be the best and safest approach to the protection of infants (15). Most evidence suggests that maternal antibody acquired by the fetus through the placenta or by neonates through breast milk has a protective effect on neonates during the first 4 to 8 weeks of life (16–25). Thus, the goal of the maternal immunization strategy is to increase matAbs in neonates to levels that will extend the time of infant protection after birth.

We have developed novel virus-like particle (VLP) vaccine candidates for RSV (26–30). In contrast to soluble proteins, VLPs robustly stimulate immune responses without the complications of adjuvant addition (15, 26, 27, 31). Because the production of VLPs does not require viral replication, multiple antigens and different conformational forms of antigens can be assembled into VLPs, in contrast to attenuated viruses, which must remain infectious. VLPs are also safer as vaccines for many populations, such as the very young, than infectious, attenuated, or vector virus-based vaccines since they do not contain a genome and do not produce a spreading infection.

McLellan et al. have identified mutations in the RSV F protein (DS-Cav1 F mutant) that stabilize the prefusion conformation (9). This prototype prefusion F protein is now widely used in many vaccine candidates at different stages of development. Indeed, we have constructed VLPs containing this stabilized prefusion RSV F protein together with the RSV G protein and have established the superiority of the DS-Cav1 F-containing VLPs over post-F protein- or wild-type F protein-containing VLPs in inducing NAbs in both mice and cotton rats (15, 26, 27). Furthermore, we have tested the efficacy of these DS-Cav1 F VLPs in a cotton rat model of maternal immunization, comparing the responses to these DS-Cav1 F VLPs to those to a postfusion F VLP as well as soluble versions of the pre- and postfusion F proteins (15). We reported that the immunization of dams during pregnancy with pre-F VLPs resulted in improved protection of the offspring of these animals from RSV challenge compared to the protection achieved with post-F VLPs or soluble F proteins (15).

Other investigators have described alternative stabilized prefusion F proteins with mutations different from those in the DS-Cav1 F protein (32–37). We have recently reported that VLPs assembled with five different versions of mutation-stabilized prefusion RSV F proteins can differentially bind monoclonal antibodies (MAbs) specific to the prefusion F protein, a result indicating differences in the conformation of these prefusion F proteins (38). Furthermore, the different VLP-associated pre-F proteins differentially impacted immunogenicity, with two of them resulting in significantly improved titers of neutralizing antibodies (NAbs) in mice compared to those achieved with DS-Cav1 F-containing VLPs. With the goal of identifying pre-F VLPs with improved efficacy as maternal vaccines, we compared the responses to VLPs containing these two alternative versions of prefusion F with those to DS-Cav1 F VLPs. We report here that VLPs containing these alternative prefusion F proteins are more effective as a maternal vaccine in cotton rat dams than the DS-Cav1 F VLPs, resulting in significantly better protection of neonates from RSV challenge and lower pathogenicity.

RESULTS

Characterization and validation of VLP stocks. The VLPs used as immunogens were based on the core proteins of the Newcastle disease virus (NDV) M and NP proteins and contained the RSV F and G glycoproteins (26, 27, 29). The RSV proteins were assembled into the VLPs as chimera proteins, with the sequences of the ectodomains of the RSV F and G glycoproteins being fused to the transmembrane (TM) and cytoplasmic (CT) domains of the NDV F and HN proteins, respectively. Four different VLPs were prepared, with VLP each containing the same G chimera protein but a different mutant F chimera protein. One F protein contained the DS-Cav1 prefusion F protein, while one contained the post-F protein as a control, as previously described (27, 38). A third VLP contained an F protein with the cleavage sites and intervening p27 sequences replaced with a 7-amino-acid GS-rich linker sequence as well as two point mutations, N67I and S215P (here named UC-2 F). This mutant was the SC-DM mutant described by Krarup et al. (36). The fourth VLP contained an F protein with the same changes as in UC-2 F but with an additional point mutation, D486N, similar to the SC-TM mutant described by Krarup et al. (36), and here named UC-3 F. The three prefusion F proteins also contained the foldon sequence inserted between the RSV F protein ectodomain and the NDV F protein transmembrane domain to further stabilize the prefusion conformation (38).

The VLPs were prepared by transfecting avian cells with cDNAs encoding the NDV M and NP proteins, the G protein chimera, and one of the mutant F chimera proteins (DS-Cav1 F, UC-2 F, UC-3 F, or post-F). VLPs released into the cell supernatant were purified as previously described (27, 38, 39), and the F protein content of the purified VLPs was quantified by Western blotting (using an anti-RSV HR2 peptide antibody) and characterized by monoclonal antibody (MAb) binding to the VLPs as previously described (27, 38, 39). The VLP stocks used in this study were adjusted for equivalent levels of F protein as described previously and shown by Cullen et al. (38). The prefusion conformation of the F protein in the VLPs was validated by binding prefusion F-specific MAbs D25 and AM14 to the VLPs as previously reported (38).

Infection and immunization. For comparisons of the efficacy of the three VLPs containing alternative prefusion F proteins as maternal vaccine candidates, female cotton rats were first infected intranasally with the RSV A/Long strain (primed) in order to mimic the immunological condition of the majority of the adult human population (Fig. 1). After 8 weeks, the animals were bred and then different groups were immunized at 1, 2, or 3 weeks of gestation (cotton rat gestation is 4 weeks) with the different VLPs (Fig. 1) in order to determine the optimal time for the transfer of protective antibodies to the offspring. Serum samples were acquired before gestation (8 weeks after priming), at 4 weeks of gestation just prior to offspring delivery, and after gestation to monitor the neutralizing antibody responses as well as total anti-F antibodies in the dams and the durability of these antibodies. Serum samples were acquired from the offspring of these dams at 4 weeks after birth to assess the serum

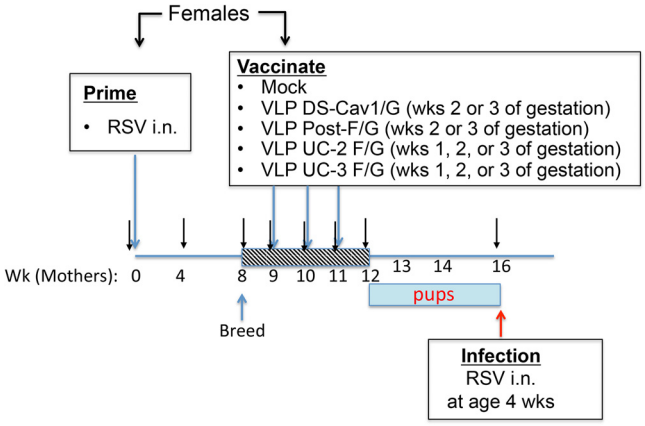

FIG 1 VLP immunization of RSV-primed cotton rats. Diagram of animal protocol. Female cotton rats were primed by intranasal (i.n.) infection with RSV A/Long (10^5 PFU/animal) on day 0. Eight weeks later, all the females were set up in breeding pairs. Immunizations were performed as follows (downward blue arrows). At days 63, 70, and 77, different groups of females were immunized with UC-2 F or UC-3 F VLPs or PBS. In addition, on days 70 and 77, other groups of females were immunized with DS-Cav1 F or post-F VLPs. A group of uninfected females was maintained as controls. Pup delivery began at week 12. Pups were challenged intranasally with RSV A/Long (10^5 PFU/pup) at 4 weeks after delivery (~week 16; red arrow) and sacrificed 4 days later for determination of virus titer in lungs and nasal tissue, as well as cytokine gene expression in lungs. Serum samples were acquired throughout at the times indicated by downward black arrows. RSV infections indicated as Prime and Infection; VLP immunizations indicated in Vaccinate box.

NAbs and total anti-F antibodies acquired from the dams (Fig. 1). The offspring were challenged with RSV (RSV A/Long strain) at 4 weeks of age by intranasal inoculation, and at 4 days after challenge, the pups were sacrificed for determination of the titers of RSV in the lungs and the nasal tissue, as well as cytokine mRNA levels and lung histology, important parameters for assessing protection from challenge and safety.

NAbs and total anti-F protein IgG induced by VLPs with three different versions of the prefusion F protein. Having identified two versions of prefusion F VLPs (UC-2 F and UC-3 F VLPs) that stimulated significantly higher NAb titers than DS-Cav1 F VLPs in mice (38), we asked if the UC-F VLPs would be more effective as maternal vaccines in cotton rats. First, we compared the immune responses in pregnant cotton rats after immunization with DS-Cav1 F, UC-2 F, and UC-3 F VLPs at different weeks of gestation. As controls, a group of animals was also immunized with postfusion F-containing VLPs (post-F VLPs). Figure 2A and Table 1 show the NAb titers in the sera obtained from these dams just before delivery, at 4 weeks of gestation. Immunization with UC-3 F VLPs at 2 weeks of gestation resulted in higher NAb titers than immunization with DS-Cav1 F or UC-2 F VLPs; however, the differences did not reach significance. The NAb titers in animals immunized at 3 weeks of gestation with UC-3 F VLPs were significantly higher than those after DS-Cav1 F VLP immunization ($P < 0.01$), and the NAb titers in animals immunized with post-F VLPs were significantly lower ($P < 0.0001$) than those in UC-3 F-immunized rats. Interestingly, the titers obtained with each VLP tended to be lower after immunization at 3 weeks of gestation than the titers induced by the same VLP at 2 weeks of gestation, although the differences were statistically significant only for the post-F VLPs ($P < 0.0001$). Lower levels of antibodies in animals immunized at 3 weeks of gestation may reflect an enhanced rate of transfer of antibodies transplacentally to the offspring close to the delivery time. NAb titers after UC-2 F VLP or UC-3 F VLP immunization at 1 week of gestation were lower than the titers in animals after immunization at 2 and 3 weeks of gestation, suggesting that immunizations at later times during gestation are more optimal. In all dams immunized with a prefusion F VLP, the NAb titers were higher than those after post-F VLP immunization. These results show that immunization with UC-3 F VLPs at 2 and 3 weeks of gestation yielded the highest NAb titers in pregnant dams.

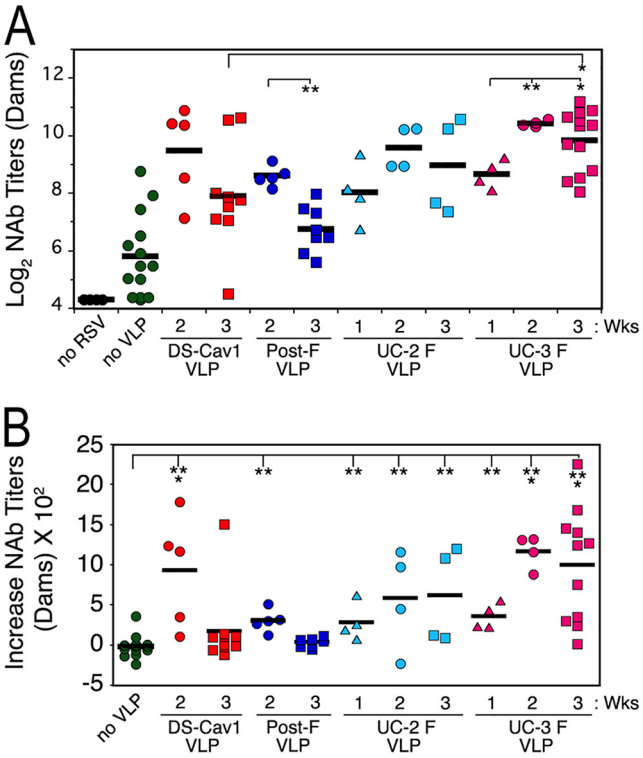

FIG 2 Neutralizing antibody (NAb) titers in pregnant dams. (A) Serum samples were acquired at day ≥84 (before pup delivery) from all groups of dams immunized with the four VLPs or PBS at 1, 2, or 3 weeks of gestation. Each symbol represents the NAb titer in an individual animal. Titers are the $\log_2$ value of the dilution of sera that inhibited RSV in a plaque reduction assay by 60%. The means are shown as bars. A comparison of the NAbs between the indicated groups was performed by one-way ANOVA followed by the Tukey *post hoc* test. *, $P < 0.05$; **, $P < 0.005$. (B) Increase in RSV NAb titers by vaccination of RSV-primed cotton rats. Serum samples obtained from dams before vaccination and subsequently before delivery were compared to determine the change in the NAb response (serum NAb titer before delivery minus serum NAb titer before vaccination) for each vaccine preparation administered to RSV-primed dams. Each symbol represents one dam, and the bar for each group represents the mean value. The increase in the titers of NAbs achieved between the respective mock- and VLP-vaccinated groups was compared by one-way ANOVA followed by the Tukey *post hoc* test. **, $P < 0.005$; ***, $P < 0.0001$.

The total increases in NAb titers achieved by vaccination of each dam in the groups were calculated by subtracting the titers of NAbs before vaccination from the titers of NAbs before delivery of the offspring and are shown in Fig. 2B. This parameter measures the boosting capacity that each vaccine has in RSV-primed dams, an indication of vaccine potency in RSV-seropositive animals. All VLP immunizations increased the RSV NAbs titers, but immunization with DS-Cav1 F at 2 weeks of gestation and with UC-3 F VLPs at both 2 and 3 weeks of gestation was significantly more effective than the immunizations with the other VLPs.

To determine if the differences in NAb titers observed were reflected in the total anti-F antibodies induced by the different VLPs, the total anti-pre-F binding IgG and total anti-post-F binding IgG were measured by enzyme-linked immunosorbent assay (ELISA) using soluble prefusion F protein (DS-Cav1 F) and soluble post-F protein as targets (Fig. 3, left and right, respectively). As for the NAb titers, the total anti-pre-F binding antibody levels were significantly lower after immunization with all VLPs at 3 weeks of gestation than the levels obtained after immunization with the same VLP at 2 weeks of gestation. The titers of pre-F binding antibodies in the sera of dams immunized at 3 weeks of gestation were significantly different after DS-Cav1 F, UC-2 F, and UC-3 F immunization, with the titers in the sera of UC-3 F VLP-vaccinated dams being lower than the titers in the sera of the other groups. These drops in titers between the immunizations at 2 and 3 weeks were not observed for anti-post-F binding antibodies. Thus, the differences between anti-pre-F binding antibodies and anti-post-F

TABLE 1 Representation of the immunization groups for female cotton rats[d]

Group	Nomenclature	No. of dams	No. of pups	Mean no. of pups/litter	Pup mean NAb titer[b]	Pup mean VT[c]	
						Lung	Nose
Naive unvaccinated	(−)	ω	41		4.32 ± 0	5.1 ± 0.4	5.4 ± 0.4
RSV unvaccinated	PBS	14	58	4	4.32 ± 0.07	4.7 ± 0.4	5.2 ± 0.4
RSV DS-Cav1 F vaccinated (wk 2)	DS-Cav1 2 VLP	5	16	4[a]	5.5 ± 0.3	3.3 ± 0.3	4.4 ± 0.4
RSV DS-Cav1 F vaccinated (wk 3)	DS-Cav1 3 VLP	10	53	5.3	5.5 ± 0.2	4.2 ± 0.4	4.8 ± 0.4
RSV post-F vaccinated (wk 2)	Post-F 2 VLP	5	23	4.6	4.4 ± 0.05	3.6 ± 0.3	4.6 ± 0.4
RSV post-F vaccinated (wk 3)	Post-F 3 VLP	9	53	5.8	5.4 ± 0.1	3.3 ± 0.3	4.8 ± 0.4
RSV UC-2 vaccinated (wk 1)	UC-2 1 VLP	4	23	5.8	5 ± 0.2	3.9 ± 0.3	4.6 ± 0.4
RSV UC-2 vaccinated (wk 2)	UC-2 2 VLP	4	16	4	4.5 ± 0.1	3.4 ± 0.3	4.5 ± 0.4
RSV UC-2 vaccinated (wk 3)	UC-2 3 VLP	4	29	7.2	5.2 ± 0.2	2.5 +/−0.2	4.6 ± 0.3
RSV UC-3 vaccinated (wk 1)	UC-3 1 VLP	4	19	6.3[a]	6.1 ± 0.5	3.5 ± 0.3	4.7 ± 0.4
RSV UC-3 vaccinated (wk 2)	UC-3 2 VLP	4	16	4	6.1 ± 0.2	2.5 ± 0.2	4.0 ± 0.3
RSV UC-3 vaccinated (wk 3)	UC-3 3 VLP	14	78	5.5	7.5 ± 0.2	2.6 ± 0.2	4.3 ± 0.5

[a]One dam in the group did not give birth.
[b]Log_2 NAb titer.
[c]Log_{10} RSV titers (VT; gram per tissue).
[d]The number of dams, the number of pups delivered in each group, the mean number of pups per litter delivered, the mean titer of the NAbs, and the lung and nasal tissue viral titers are included. ω, pups obtained from the SBI breeding facility.

binding antibodies in dams just before delivery suggest a selective transfer of antibodies to the offspring that is biased toward an increased transfer of pre-F binding antibodies (Fig. 3).

Durability of RSV immune responses in dams. There is evidence that the majority of disease in infants and young children is due to transmission of the virus from infected family members (40). Thus, a major element in the protection of infants and young children from RSV disease is the prevention of infection in family members. To assess the durability of the anti-RSV antibodies induced in dams after VLP immunization, the NAb titers and total anti-pre-F binding and anti-post-F binding antibodies in

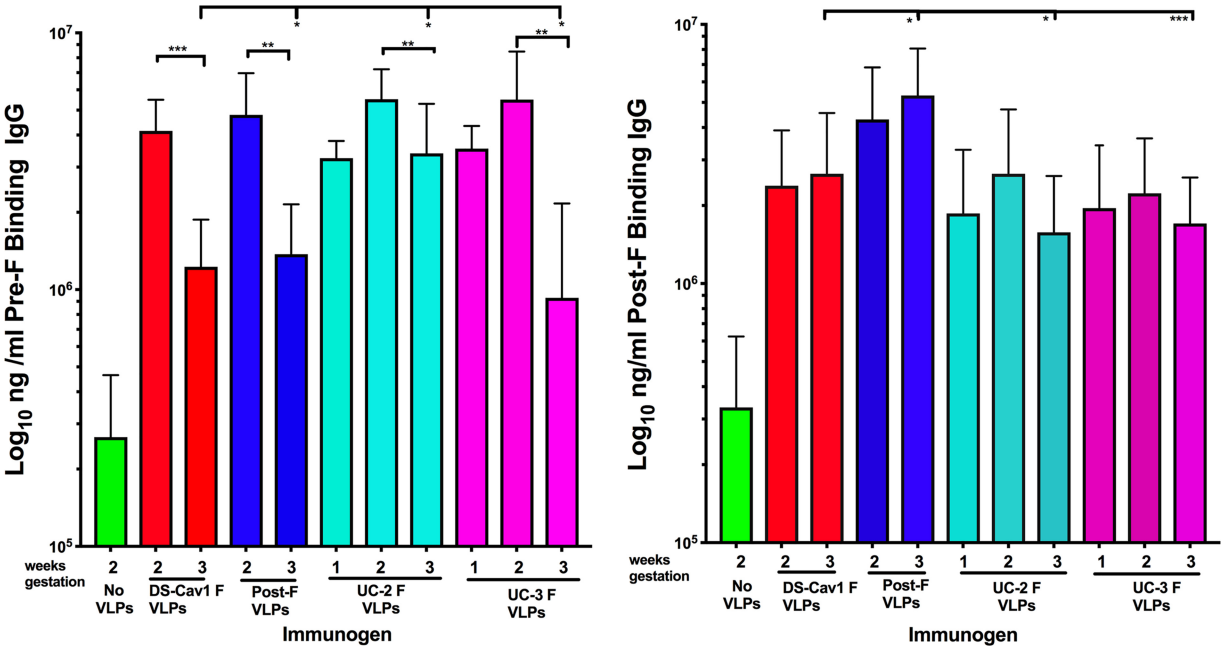

FIG 3 Total anti-F antibodies in the sera of pregnant dams. The concentrations (in nanograms per milliliter) of total anti-pre-F binding IgG antibodies (left) and anti-post-F binding IgG antibodies (right) in pooled sera acquired at day 84 were determined by ELISA using purified soluble DS-Cav1 pre-F protein or soluble post-F protein as targets. Sera from the dams in each group were pooled, and the data show the means and standard deviations from three to five separate determinations of the concentrations (in nanograms per milliliter) in each pool. The titers between the sera of dams immunized at 2 and 3 of gestation weeks with each VLP were compared by Student's *t* test. **, $P < 0.005$; ***, $P < 0.0005$. The titers between dams receiving the DS-Cav1 F VLP immunization at 3 weeks of gestation and dams receiving the other VLP immunizations at 3 weeks of gestation were compared by one-way ANOVA. *, $P < 0.05$; ***, $P < 0.0005$.

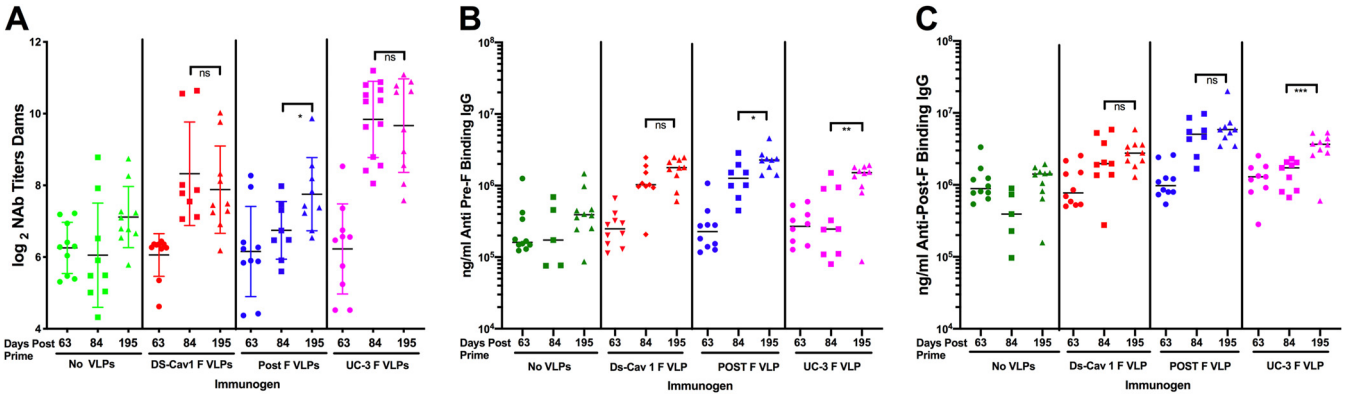

FIG 4 Durability of neutralizing and total anti-F antibody titers in female cotton rats. (A) NAb titers in dams at 63, 84, and 195 days after RSV priming and immunization with DS-Cav1 F, post-F, or UC-3F VLPs at 3 weeks of gestation. The titers of the individual dams are shown. The means and standard deviations of the titers are shown. (B and C) The concentrations (in nanograms per milliliter) of anti-F IgG that bind to soluble pre-F proteins (B) or soluble post-F proteins (C) in individual dams at 63, 84, and 195 days after RSV priming and immunization with DS-Cav1 F, post-F, or UC-3 F VLPs at 3 weeks of gestation. The means and standard deviations of the concentrations (in nanograms per milliliter) are shown. The results for sera harvested at 84 and 195 days for each VLP were compared by Student's *t* test. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$; ns, not significant.

individual dams were measured at 64 days (before immunization), 84 days (before delivery), and 195 days (111 days after delivery) after the RSV prime (infection). These assays focused on dams immunized with DS-Cav1 F VLPs, post-F VLPs, and UC-3 F VLPs at 3 weeks of gestation. Figure 4A shows that the NAb titers in all animals at 195 days were similar to the titers or increased compared to the titers obtained with the same VLP just before delivery at 84 days post-RSV prime. Similarly, the total pre-F binding antibodies and total post-F binding antibodies in each group did not diminish with time (Fig. 4B and C). Thus, anti-RSV F antibodies in dams do not decrease with time after VLP immunization, suggesting that the VLPs effectively stimulate cotton rat anti-F protein B cell memory and long-lived anti-F protein-secreting bone marrow-associated plasma cells induced by RSV priming, as we have previously suggested in murine studies (41).

Serum titers in offspring of immunized dams. To assess the transfer of antibodies to pups after maternal immunization with the different prefusion F VLPs, the neutralization titers and total anti-F IgG levels were measured in the pup sera at 4 weeks after birth. We have previously shown that this is the time when low to undetectable levels of maternal NAbs remain in circulation in offspring from RSV-primed, unvaccinated mothers (42). Figure 5A shows the NAb titers in individual pups, while in Fig. 5B, each symbol shows the mean titers in pups from the same litter (Table 1 shows a sum of the data). The results show that the average NAb titers in the pup sera did not directly correspond to the NAb titers in the dams. In contrast to the titers in the sera from the dams, the NAb titers in sera from the offspring of dams immunized at 3 weeks gestation were similar to or higher than those in sera from the offspring of dams immunized at 2 weeks of gestation. The titers in the offspring of dams immunized with UC-3 F VLPs at 3 weeks of gestation were the highest and approximately 1 $\log_2$ higher than the titers in the offspring of dams immunized with DS-Cav1 F VLPs. The titers of NAbs in sera from the offspring of UC-2 F VLP-immunized dams were low and comparable to the titers in sera from the offspring of post-F VLP-immunized dams. Thus, UC-3 F VLP immunization of dams at 3 weeks of gestation resulted in the highest serum NAb titers in their offspring.

Figure 6A and B show the titers of total anti-pre-F binding antibodies and anti-post-F binding antibodies in pools of pup sera from each litter. Interestingly, in contrast to the dam sera, the levels of total anti-pre-F binding antibodies in the pup sera were not significantly different after immunization of their dams with the four different VLPs, nor did the time of immunization of the dams during gestation significantly affect the levels of total anti-pre-F binding antibodies in their offspring. However, there were significant

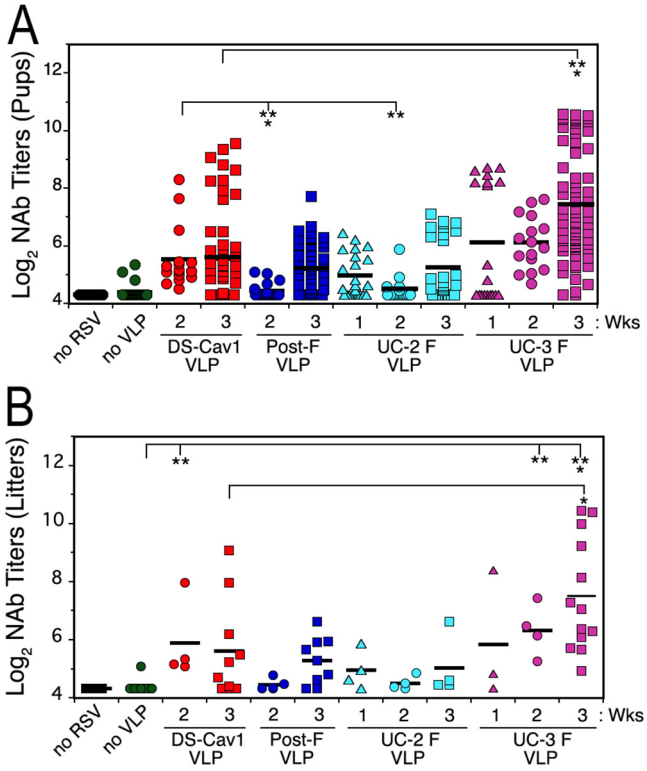

FIG 5 NAb titers in pup sera. (A) Titers are the $\log_2$ value of the dilution of pup sera harvested 4 weeks after birth that resulted in 60% inhibition of RSV in a standard plaque reduction assay. Each symbol represents the titer in an individual pup. The increase in the titers of NAbs achieved between the respective mock-vaccinated and VLP-vaccinated groups was compared by one-way ANOVA followed by the Tukey *post hoc* test. **, $P < 0.005$; ***, $P < 0.0001$. (B) Average titers of NAbs in the sera of the offspring of a single dam are shown. The increase in the titers of NAb achieved between the respective mock-vaccinated and VLP-vaccinated groups was compared by one-way ANOVA followed by the Tukey *post hoc* test. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0001$.

differences in the levels of post-F binding IgG in the sera of the offspring of dams immunized with the different prefusion F VLPs, and the time of immunization of the dams differentially affected the levels of antibodies recognizing post-F protein in the pups' sera. The titers of post-F binding antibodies in sera from dams immunized at 3 weeks of gestation were significantly higher than the titers in the sera from dams immunized at 2 weeks of gestation, with the exception of the UC-2 F VLP-immunized dams, in which the titers did not seem to change with the time of vaccination.

Protection of pups from RSV challenge. To assess the level of protection of pups from RSV challenge afforded by immunization of dams, the titers of virus in the lungs (Fig. 7A) and nasal tissue (Fig. 7C) of the pups were measured at 4 days after challenge with the RSV A/Long strain. Figures 7B and D show the average titers in the lungs and nasal tissue, respectively, in the pups from each litter. As we have previously reported, protection from RSV replication in lungs and noses was not very different after immunization with the DS-Cav1 F or post-F VLPs. The times of immunization of the dams with DS-Cav1 F or post-F VLPs had no differential effects on improved lung or nasal protection. Immunization of the dams at 1 or 2 weeks of gestation with UC-2 F VLPs resulted in similar levels of protection as immunization of the dams with DS-Cav1 F VLPs, but immunization at 3 weeks of gestation significantly improved lung protection. Striking, however, was the level of protection observed after immunization of the dams with UC-3 F VLPs. Immunization of the dams with this VLP at either 2 or 3 weeks of gestation decreased the lung titers by 6- and 40-fold, respectively, compared to the titers obtained from the pups of dams immunized with DS-Cav1 F VLPs at 2 or 3 weeks (Fig. 7A and B; Table 1).

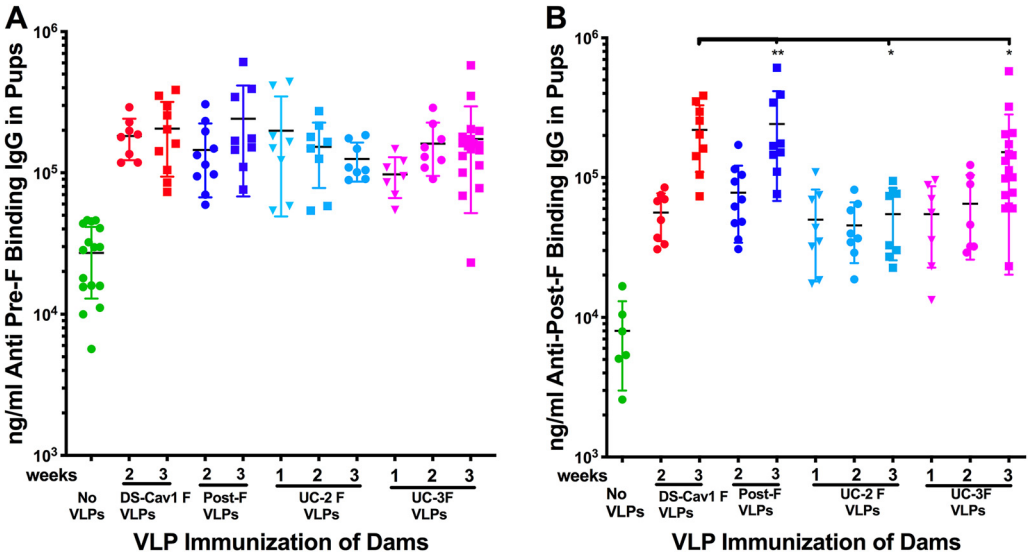

FIG 6 Total anti-F antibodies in the sera of pups. Sera were acquired at 4 weeks after birth. The sera of offspring of a single dam were pooled, and each point is the average of two separate determinations of the concentrations (in nanograms per milliliter) of anti-F antibody in each pool of sera. The concentrations (in nanograms per milliliter) of anti-pre-F binding IgG (A) or anti-post-F binding IgG (B) in pup sera were determined by ELISA using purified soluble DS-Cav1 pre-F protein or soluble post-F protein as the target. The mean and standard errors are shown. There were no significant differences between the concentrations (in nanograms per milliliter) of anti-pre-F binding IgG. The concentrations (in nanograms per milliliter) of anti-post F binding IgG between the DS-Cav1 F VLP immunization at 3 weeks of gestation and the other VLP immunizations at 3 weeks were compared by one-way ANOVA. *, $P < 0.05$; **, $P < 0.005$.

Increased protection from RSV replication in nasal tissue was also significantly improved by immunization of the dams with UC-3 F VLP. The nose titers obtained in the offspring of dams immunized with UC-3 F VLP at 2 or 3 weeks of gestation were reduced by 3- to 4-fold compared to the titers in the offspring of dams immunized with DS-Cav1 F VLPs at 2 or 3 weeks of gestation (Fig. 7C and D). Thus, maternal immunization with UC-3 F VLPs significantly improved the protection of the offspring from RSV challenge compared to immunization with DS-Cav1 F VLPs. Similar results were achieved when the mean titers of the litters were considered for the estimation (Table 1).

Safety of VLP immunization. To assess the safety of VLP-induced maternal antibodies in the offspring, the extent of lung pathology was assessed (by scoring in a blind manner) in the pups after RSV challenges. Figure 8 shows the relative levels of peribronchiolitis, perivasculitis, interstitial pneumonia, and alveolitis in the lungs of pups sacrificed 4 days after RSV challenge. Most of the pathology scores in pups from vaccinated dams were significantly lower than those in pups from RSV-primed, mock-vaccinated dams, as exemplified in the representative lung histology fields shown in Fig. 9. Furthermore, the scores, particularly those measuring peribronchiolitis and perivasculitis, were lower in the offspring of dams immunized with UC-3 F VLPs and UC-2 F VLPs than in the offspring of dams immunized with DS-Cav1 F VLPs, indicating that the UC F VLP immunization of dams resulted in lower inflammatory responses in RSV-challenged offspring than DS-Cav1 F VLP immunization.

The levels of gamma interferon (IFN-γ), interleukin-6 (IL-6), and IL-4 cytokine mRNA expression in lungs were also determined as an additional measure of the inflammatory response after RSV challenge of the pups (Fig. 10). The cytokine mRNA expression levels in the offspring of dams primed and immunized with all VLPs were significantly lower than those in the offspring of dams that were RSV primed but mock vaccinated, indicating a reduction of the overall lung inflammatory response by VLP immunization.

Monoclonal antibody binding to DS-Cav1 F VLPs and UC-3 F VLPs. The differences between the presentation of prefusion-specific epitopes on the DS-Cav1 F VLPs and the UC-3 F VLPs could account for the differences in protection of the offspring of

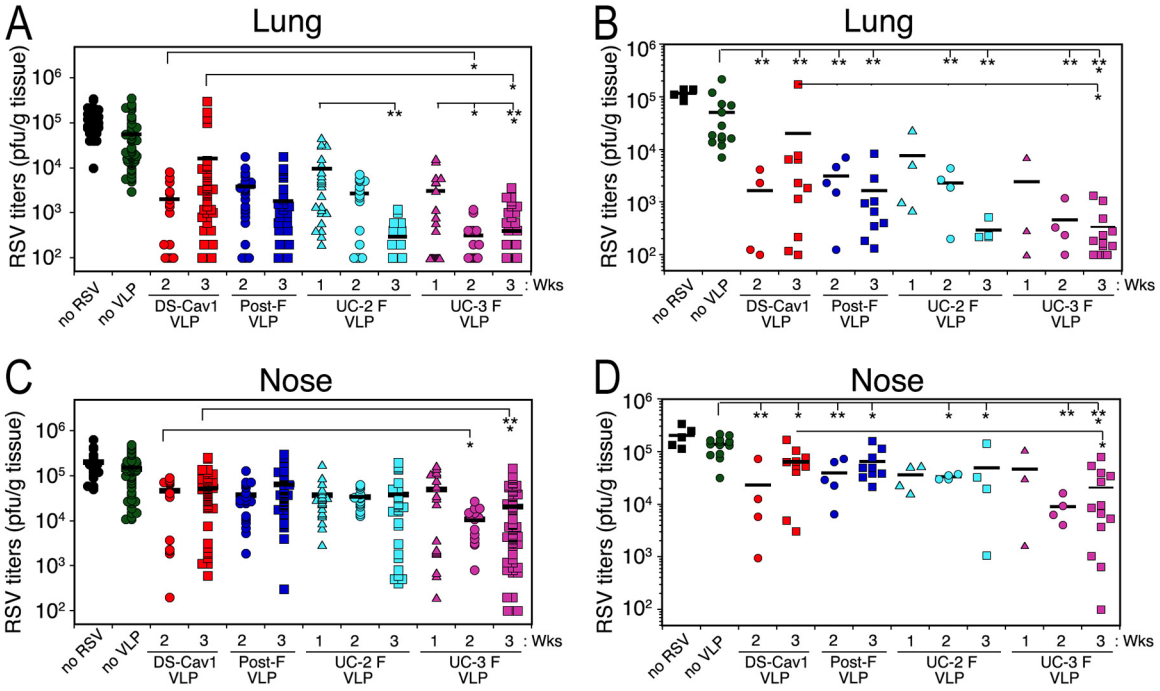

FIG 7 Virus titer in lung and nasal tissue of challenged pups. (A and B) The lungs (A) and nasal tissue (B) were harvested 4 days after an RSV challenge and the RSV titer per gram of tissue was determined in a plaque assay. Results from the tissue of individual pups are shown, with the mean indicated as a bar. The protection achieved between the respective mock-vaccinated group and the groups vaccinated intramuscularly with VLP or RSV was compared by one-way ANOVA followed by the Tukey *post hoc* test. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0001$. (C and D) Average titers of virus in lungs (C) or nasal tissue (D) in the offspring of a single dam. The protection achieved between the respective mock-vaccinated group and the groups vaccinated intramuscularly with VLP or RSV was compared by one-way ANOVA followed by the Tukey *post hoc* test. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0001$.

dams immunized with the two VLPs. Previous characterization of MAb binding to these VLPs has suggested such a difference (38). To extend these results, we quantified the binding of MAb to these two VLPs under conditions where MAb binding was saturating. VLPs with the same concentrations of F protein were bound to microtiter wells, and increasing concentrations of representative MAb were bound to the wells. Figure 11 shows that UC-3 F VLPs bound more prefusion-specific MAbs D25 and AM14 (43) at saturation (Fig. 11B and C) than DS-Cav1 F VLPs, although the binding of the MAb motavizumab (Fig. 11A), a site IIb antibody (43, 44), saturated at similar levels of binding. However, the binding of palivizumab, a site IIa MAb (44), to DS-Cav1 F VLPs was slightly less than the binding to UC-3 F VLPs. In contrast, the binding of site I- and site IV-specific antibodies MAb 1112 and MAb 1243, respectively (45), specific to both prefusion and postfusion F protein, was higher on DS-Cav1 F VLPs than on UC-3 F VLPs, further indicating conformational differences between the two prefusion F proteins. Figure 11 also shows that the length of storage of VLPs at −80°C has no effect on the relative binding of MAbs D25 and AM14 to DS-Cav1 F VLPs and UC-3F VLPs (Fig. 11G to I).

DISCUSSION

Cotton rats are widely used as an animal model for exploring parameters for effective immunization with RSV vaccine candidates, including maternal vaccines for protection of offspring from RSV disease. These animals are permissive for RSV and are therefore an excellent model for assessing protection from RSV infection (46). Enhanced respiratory disease (ERD) upon RSV challenge can be demonstrated in these animals after immunization with FI-RSV, making them a good system to address the safety of vaccine candidates (46). In addition, this model has proven to be clinically relevant to determine the efficacy of immunoprophylaxis against RSV, predict the dose of antibodies required for efficacy in humans, and predict the lack of efficacy of immuno-

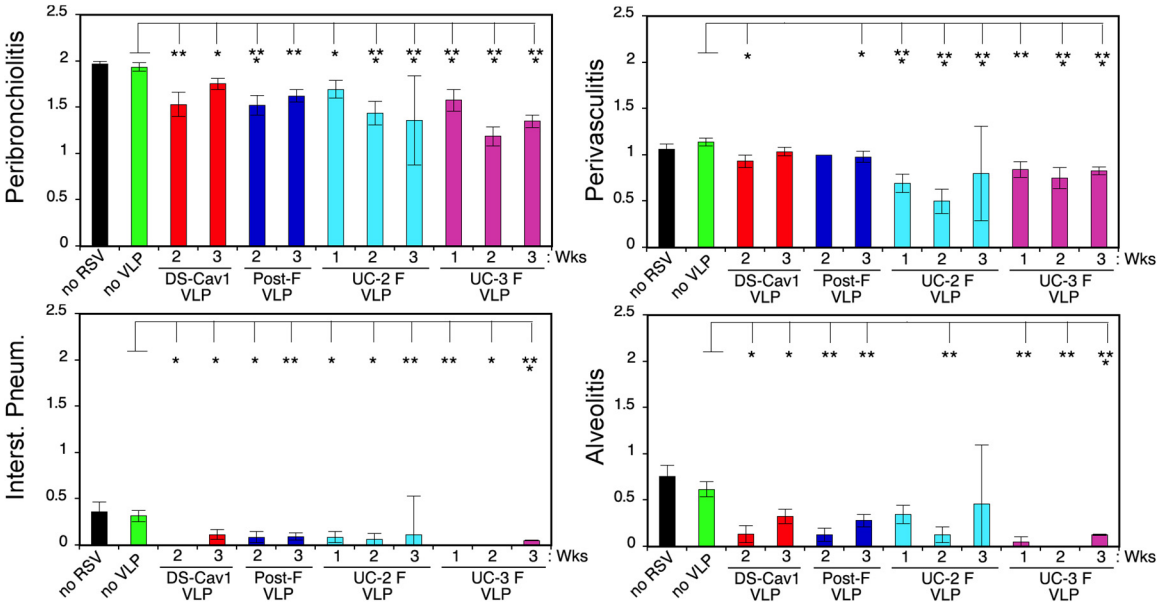

FIG 8 Lung pathology in offspring. The lung histopathology scores in RSV-infected litters born to RSV-primed dams that were vaccinated once during pregnancy are indicated. The bars represent the mean. The group labeled "no RSV" represents animals born to RSV-naive mothers, used as a positive control. The histopathology between the mock-vaccinated (PBS) and the test-vaccinated animals was compared by ANOVA followed by the Tukey *post hoc* test. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0001$. Interst. Pneum, interstitial pneumonia.

therapy against RSV (39). However, the use of cotton rats to assess the maternal transfer of immunity may encounter some predictive limitations due to possible differences in the rate of antibody transfer through the placenta and breast milk in cotton rats versus humans (21–25).

Using this animal model, the efficacy of RSV VLP vaccine candidates has been addressed, showing that VLPs assembled with the prefusion DS-Cav1 F protein induced NAb titers superior to those induced by post-F VLPs or RSV infection (26). Furthermore, their efficacy as a maternal vaccine for the protection of offspring from RSV challenge has been addressed (15). The study described in this report extends those studies by focusing on the role of the F protein conformation in efficacy as a maternal vaccine, the safety of different vaccine candidates for the offspring of immunized dams, and the durability of the responses to vaccine candidates in dams.

Since the initial report of the DS-Cav1 F protein mutations, other investigators have identified different mutations that stabilize the prefusion F protein, as determined by reactivity to MAbs specific to the prefusion F protein (32–37). We have shown that prefusion F-specific monoclonal antibodies bind to five different mutant prefusion F proteins to different extents (38). We found that the populations of antibodies induced in mice by the different pre-F VLPs were different, as determined by their abilities to compete for the binding of MAbs to pre-F protein (38). Further, we found that the levels of NAbs induced in mice by two different prefusion F VLPs were two to three times higher than the levels induced by DS-Cav1 F VLPs (38). Taken together, our results indicate that different mutation-stabilized prefusion F proteins differ in conformation and antigenicity and emphasize the importance of the identification of the optimal conformation for the induction of protective antibodies.

The results presented here show that different mutation-stabilized pre-F proteins may vary in their efficacy as a maternal vaccine. Our results show that, like in the murine studies, immunization with UC-3 F VLPs resulted in higher NAb titers in pregnant cotton rats than immunization with DS-Cav1 F VLPs. However, UC-2 F VLP immunization resulted in titers similar to those resulting from DS-Cav1 F VLP immunization. Most importantly, the NAb titers in the serum of the offspring of the UC-3 F VLP-immunized dams were significantly higher than those in the serum of the offspring of animals

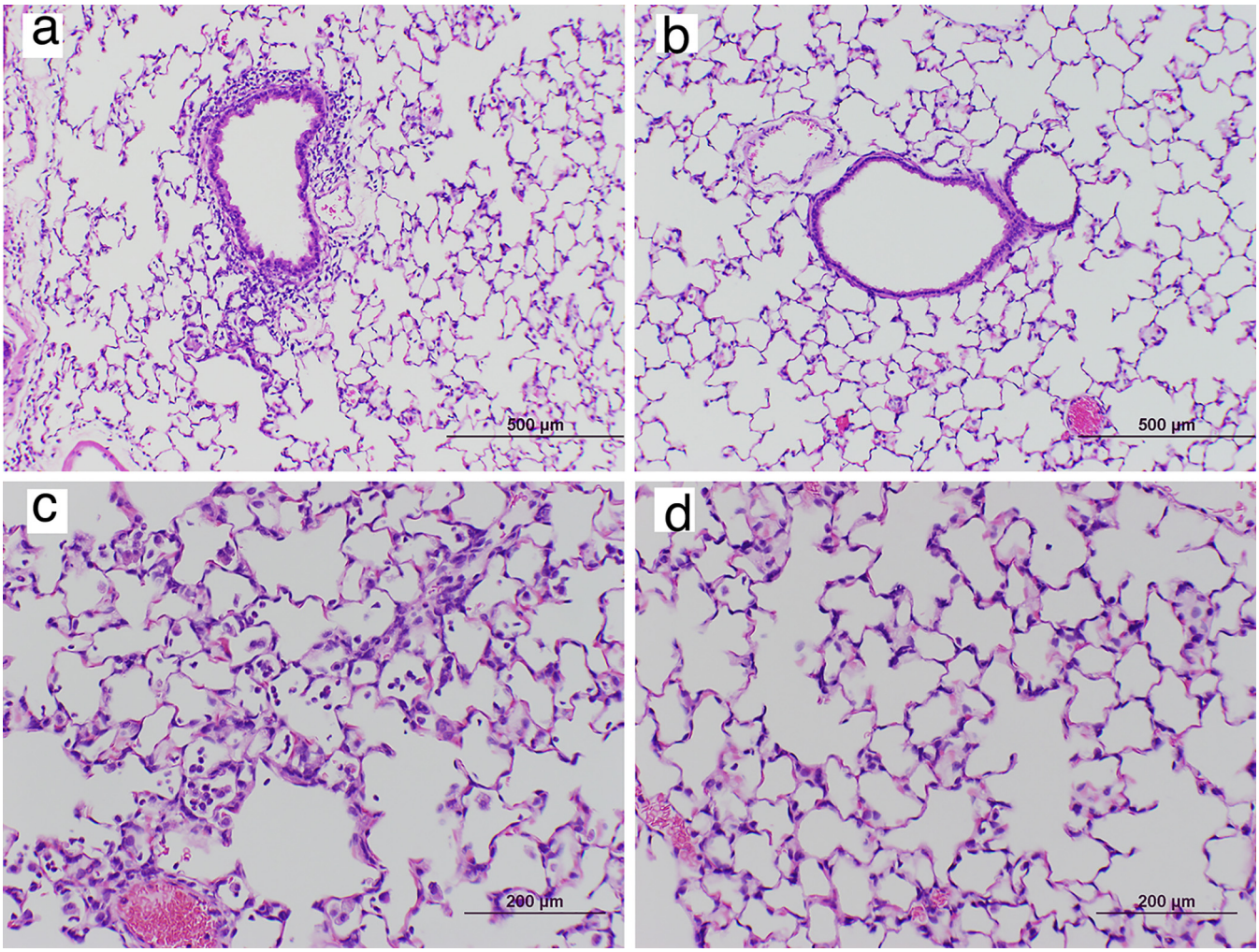

FIG 9 Lung histology in offspring. Representative lung histologies from 4-week-old juveniles born from control unvaccinated dams (a and c) or from UC-3 F VLP-vaccinated dams (b and d) and challenged with RSV are shown. Magnifications, ×100 (a and b) and ×200 (c and d).

immunized with DS-Cav1 F VLPs. This increase in NAb titers translated to significantly increased protection of the offspring from RSV challenge. The virus titers in the lungs of the challenged offspring were 6- to 40-fold lower than the titers in the lungs of the offspring of DS-Cav1 F VLP-immunized dams. The titers of virus in nasal tissue were also significantly lower.

In cotton rats, we have previously defined the threshold period of 4 weeks postbirth to be the time when the protection imparted by RSV-primed dams goes from strong to negligible (42). The experiments described here were designed based on these previous observations with the purpose of ranking RSV vaccine candidates in their capability to extend protection in immunologically naive pups past this 4-week threshold.

These results raise the question of why UC-3 F VLPs are superior to DS-Cav1 F or UC-2 F VLPs as a maternal vaccine in cotton rats. An explanation for the differences in the efficacies of different prefusion F VLPs may be due to differences in the protein conformation in the different mutant prefusion F proteins. We previously reported that UC-3 F VLPs bind to the prefusion-specific MAbs D25 and AM14 (43) at levels higher than DS-Cav1 F VLPs (38). Furthermore, while UC-2 F VLPs do bind MAb D25, this VLP binds MAb AM14 very poorly (38). Here, we extended this analysis by comparing the extent of the saturation of binding of MAbs D25 and AM14 to DS-Cav1 F VLPs and UC-3 F VLPs containing equivalent levels of F protein. Clearly, the binding of these two antibodies saturated at higher levels on UC-3 F VLPs than on DS-Cav1 F VLPs. These

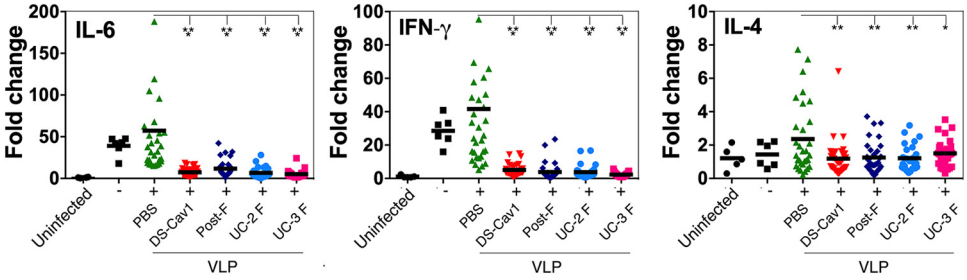

FIG 10 Lung cytokine expression. Expression of mRNA for the cytokines IL-6 (left), IFN-γ (middle), and IL-4 (right) in the lungs of RSV-infected litters ($n = 28$ to 40, randomly selected balanced by sex) born to RSV-primed dams vaccinated as indicated. The control (uninfected, $n = 4$) consisted of animals that were naive and not infected (used as a baseline for mRNA expression). Control naive (−, $n = 6$) animals were animals born to RSV-naive animals. Symbols represent individual litters. The bars represent the mean ± SEM for a group. The expression between the respective mock-vaccinated group and the groups vaccinated intramuscularly with VLP or RSV was compared by one-way ANOVA followed by the Tukey *post hoc* test. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0001$.

results suggest that while the site IIb epitope (recognized by motavizumab) was present at the same concentration on the two VLPs, the prefusion-specific epitopes recognized by D25 and AM14 are present at lower concentrations on the DS-Cav1 F VLPs than on the UC-3 F VLPs. Thus, the DS Cav1 F VLPs may stimulate lower levels of antibodies specific to these epitopes, resulting in lower levels of protection. Although several different mechanisms may be responsible for these observations, they raise the possibility that important epitopes for the induction of potent NAbs are present in different concentrations in the two versions of prefusion F protein. Lower concentrations of prefusion-specific epitopes may result in the less effective induction of potent neutralizing antibodies.

A potential reason for the differential concentrations of prefusion epitopes in DS-Cav1 F and UC-3 F VLPs is suggested by previous observations by others that the prefusion conformation of DS-Cav1 F is somewhat unstable upon storage (36, 37, 47). The instability of DS-Cav1 F was detected only after storage at 4°C for 15 to 20 days (36). Flynn et al. also reported the loss of prefusion-specific MAb D25 reactivity upon storage of DS-Cav1 F VLPs at 4°C for 102 days but not 14 days (37). They reported that storage at −70°C for 102 days had little effect on D25 reactivity. We found that different lengths of storage at −80°C had no effect on the differential levels of binding of prefusion-specific antibodies to DS-Cav1 F VLPs (Fig. 11). Furthermore, we have shown that the pre-F conformations of different pre-F proteins assembled in VLPs were stable upon incubation under a number of different conditions (38).

An alternative explanation for our results is suggested by recent analyses of prefusion influenza virus hemagglutinin (HA) and HIV Env proteins. The assumption has been made that the prefusion F protein is a single, spring-loaded metastable structure which irreversibly snaps into the low-energy postfusion state upon activation. However, this assumption should be reassessed due to recent reports of findings obtained using single-molecule fluorescence resonance energy transfer (smFRET) that the influenza virus HA (48) and the HIV Env (49–51) exist in several prefusion forms. The HA protein, prior to activation by acid pH, exists in at least three conformationally reversible intermediates. The HIV prefusion envelope protein exists in at least 3 conformations prior to the initiation of membrane fusion, a closed native form, an intermediate form, and a form bound to CD4 (50). Importantly, using smFRET, it has been shown that mutations introduced into the protein to stabilize the prefusion HIV Env protein, the SOSIP trimers (49), do not stabilize Env in its most native form but stabilize it in a conformation between the native and the CD4-bound form (50). Furthermore, the most effective, broadly neutralizing antibodies bind not to the intermediate form but to the closed native form.

Given these results with other viral fusion proteins and our finding of conformational differences in alternate versions of the mutation-stabilized RSV prefusion F

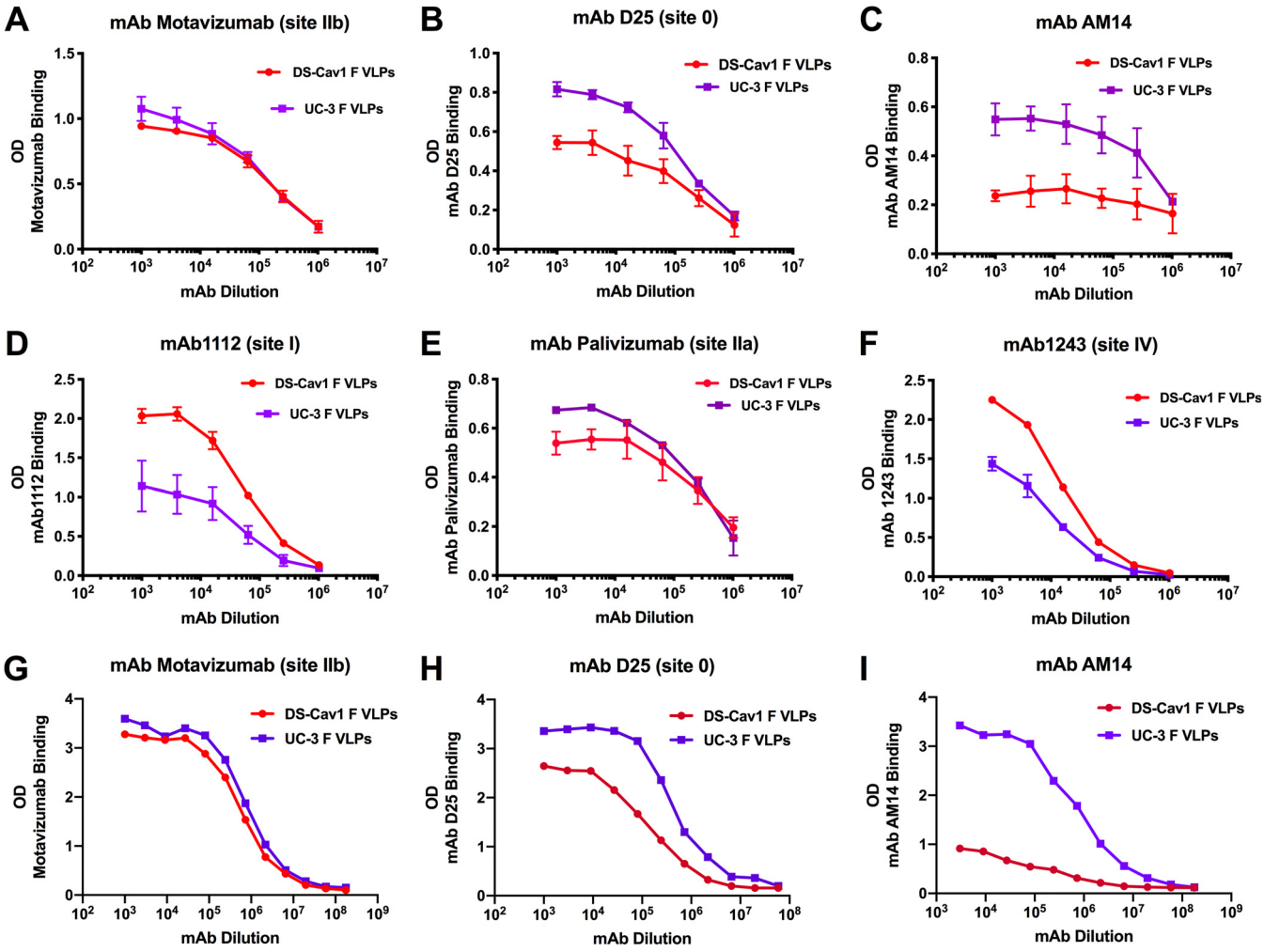

FIG 11 Relative binding of monoclonal antibodies to VLPs. (A to F) The binding, measured as the optical density (OD), of decreasing amounts of F protein-specific monoclonal antibodies to DS-Cav1 F VLPs and UC-3 F VLPs containing the same amounts of F protein is shown. VLPs were flash frozen immediately after purification and stored at −80°C. (A to F) The VLPs used in the experiments whose results are shown in the figure were stored at −80°C for 2 weeks. (G to I) Similar results were obtained with different preparations of VLPs stored at −80°C for 2 months. (A and G) Motavizumab; (B and H) prefusion-specific MAb D25; (C and I) prefusion-specific MAb AM14; (D to F) MAb 1112, palivizumab, and 1243, respectively, antibodies that bind both pre- and post-F proteins. Each point in panels A to F is the average from two separate determinations, with the standard deviation being shown.

protein, it is reasonable to hypothesize that several conformations of the RSV prefusion F may also exist and that different mutations may differentially stabilize these forms and differentially induce protective antibodies. The results of the binding of MAbs specific for sites I, IIa, and IV also indicate slight differences in the conformation of DS-Cav1 F and UC-3 F in VLPs, supporting the notion that prefusion F proteins may exist in several forms differentially stabilized by different mutations. This idea is supported by the recent report by McLellan and colleagues that site 0 exists in alternative conformations (52).

The studies reported here also addressed the importance of the time during gestation of dam immunization on the levels of protection of the neonates. We found that immunization of the dams at late stages of gestation resulted in better protection of the offspring than immunization of the dams earlier during gestation. In this context, it is important to note that the levels of NAbs in dams immunized at 3 weeks of gestation were lower than those in dams immunized earlier and that this lower level was mirrored by the total anti-pre-F binding antibodies in the sera. In contrast, the levels of NAbs in the sera of the offspring of dams immunized at 3 weeks were similar to or higher than those in the offspring of dams immunized earlier in gestation. These

results are consistent with the idea that antibodies stimulated later in gestation are generally more efficiently transferred to the offspring, reducing the levels in the dams and increasing the levels in the pups.

The safety of the different VLP immunizations of dams in the offspring upon RSV challenge was also assessed in these studies. Our previous studies of VLP immunization of adult cotton rats have previously shown no evidence of enhanced lung pathology after RSV challenge (26). Importantly, here we report that VLP immunization of dams, particularly with UC-3 F VLPs, resulted in a significant decrease in the overall lung pathology in the offspring upon their challenge with RSV compared to that seen with the other vaccines tested or compared to that seen in offspring delivered to primed, mock-vaccinated dams. The low cytokine mRNA levels in the lungs of these pups further supported this conclusion.

An important property of vaccines is the durability of protective antibodies, but many vaccines do not result in a long duration of protection. We have previously reported that VLPs with RSV glycoproteins stimulate quite durable NAbs in mice, a result that correlates with our findings in mice of significant levels of anti-F protein splenic memory B cells and anti-F protein antibody-secreting bone marrow-associated plasma cells (41). Here we have shown a similar durability of anti-F protein antibody levels in cotton rats, as the levels of NAbs and total anti-F antibodies changed little during the 118 days after VLP immunization. The durable antibody levels in mice and cotton rats are likely due to the optimal size of VLPs, defined by Bachmann and colleagues (31, 53), for induction of antibody responses. Furthermore, Bachmann and colleagues have shown that durable levels of antibodies in sera correlated with dense, highly repetitive antigens on VLP surfaces that cross-link B cell receptors, leading to the induction of durable antibodies (31).

In summary, we have shown that different mutation-stabilized prefusion F proteins in VLPs have different conformations and induce different levels of protective antibodies in pregnant cotton rat dams. We have identified a version of a prefusion VLP-associated F protein that induces significantly improved levels of protective antibodies in dams and improved protection of their offspring from RSV challenge compared to those achieved with previously tested vaccine candidates. Our results indicate that RSV VLP vaccine candidates have significant potential for use as maternal vaccines.

MATERIALS AND METHODS

Cells, virus, and plasmids. ELL-0 and HEp-2 cells, obtained from the American Type Culture Collection, were grown in Dulbecco modified Eagle medium (Invitrogen) supplemented with penicillin, streptomycin (Invitrogen), and 10% fetal calf serum (Invitrogen). Expi293F cells, obtained from Thermo Fisher/Invitrogen, were grown in Expi293 medium (Thermo Fisher/Gibco/Invitrogen). The prototype Long strain of RSV was obtained from the American Type Culture Collection (ATCC VR-26; Manassas, VA). The RSV A2 strain was obtained from Robert Finberg.

VLPs containing the RSV F and G proteins were formed with the Newcastle disease virus (NDV) core proteins NP and M. The cDNAs encoding the NDV NP and M protein have been previously described (29, 30). The RSV F and G glycoproteins were incorporated into these VLPs as chimera proteins composed of ectodomains of the F or G glycoprotein fused to the transmembrane (TM) and cytoplasmic (CT) domains of the NDV F protein or NDV HN glycoprotein, respectively, as previously described (29, 30).

The construction of genes encoding the soluble pre-F protein, the soluble post-F protein, and the soluble G protein used as the target in the ELISA was previously described (15, 27).

Polyacrylamide gel electrophoresis, silver staining, and Western blot analysis. Proteins were resolved on 8% bis-Tris gels (NuPAGE; Thermo Fisher/Invitrogen). Silver staining of the proteins in the polyacrylamide gels was accomplished as recommended by the manufacturer (Thermo Fisher/Pierce). Quantifications of NP, M, different forms of the F/F and H/G proteins, and soluble pre-F, post-F, and soluble G proteins were accomplished after their separation in polyacrylamide gels followed by silver staining or by Western blot analysis of the proteins alongside the protein standards as previously described (15, 27). For Western blot analysis, the proteins in the polyacrylamide gels were transferred to polyvinylidene difluoride membranes using dry transfer (iBlot apparatus; Thermo Fisher/Invitrogen). Proteins were detected in the blots using an anti-RSV HR2 peptide antibody, an anti-NDV F tail antibody (28, 29), or an anti-RSV G protein antibody (Thermo Fisher).

Antibodies. Monoclonal antibody (MAb) 1112 and MAb 1243 (which are specific to sites I and IV, respectively, and which were generous gifts of J. Beeler) and MAb D25 and the MAb motavizumab (which were generous gifts of J. McLellan) were used to verify the F protein conformations and for ELISA analysis

of VLPs and soluble F proteins. The anti-RSV F protein HR2 antibody and anti-NDV F-tail antibody (29), used for Western blot analyses, are polyclonal antibodies specific to the HR2 domain of the RSV F protein or the cytoplasmic tail of the NDV F protein. Anti-RSV G protein antibody is a polyclonal antibody raised against a peptide containing G protein amino acids 180 to 198 (Thermo Fisher). Secondary antibodies against goat, mouse, and rabbit IgG were purchased from Sigma.

VLP preparation, purification, and characterization. For preparation of the VLPs to be used as immunogens, ELL-0 cells growing in T-150 flasks were transfected with cDNAs encoding the NDV M protein, NP, the NDV HN/RSV G chimeric protein, and one of the F protein chimera genes as previously described (28, 29). At 24 h posttransfection, heparin (Sigma) was added to the cells at a final concentration of 10 μg/ml to inhibit the rebinding of the released VLPs to cells. At 72, 96, and 120 h posttransfection, cell supernatants were collected and VLPs were purified by sequential pelleting and sucrose gradient fractionation as previously described (28, 29). VLP preparations were flash frozen and stored at −80°C immediately after purification. Immediately prior to use as immunogens or in the ELISAs, aliquots were thawed on ice. The conformation of the F protein in the VLP preparations was verified by its reactivity to MAbs. The VLPs used here were the same as those used in the study of Cullen et al., and their full characterization is described in the report for that study (38).

Preparation of soluble F proteins. Expi293F cells were transfected with a pCAGGS vector containing sequences encoding the soluble DS-Cav1 F protein, the soluble post-F protein, or the soluble G protein as previously described (15, 38, 51). At 5 to 6 days posttransfection, total cell supernatants were collected and cell debris was removed by centrifugation. Soluble polypeptides were then purified on columns using the His tag and then the streptavidin tag as previously described (9, 15).

Quantification of soluble and VLP-associated F and G proteins. Determinations of the amounts of RSV F protein in VLPs or in soluble F protein preparations described previously (15, 38) were accomplished by Western blotting using an anti-HR2 antibody for detection and comparing the signals obtained with those on a standard curve of purified F proteins as previously described (15, 38).

Animals. *Sigmodon hispidus* cotton rats were obtained from an inbred colony maintained at Sigmovir Biosystems, Inc. (Rockville, MD). Three-week-old female cotton rats (dams) were recruited for these studies and randomly tagged and separated into the groups indicted above (Fig. 1). Eight weeks later, females were paired with males ~2 weeks older than the females for mating. Animals were prebled before inclusion in the study to rule out the possibility of preexisting antibodies against RSV. The colony was monitored for antibodies to paramyxoviruses and rodent viruses, and no such antibodies were found. All studies were conducted under applicable laws and guidelines and after approval from the Sigmovir Biosystems, Inc., Institutional Animal Care and Use Committee. Animals were housed in large polycarbonate cages and fed a standard diet of rodent chow and water *ad libitum*. All cotton rats born as a result of breeding during these studies were used for RSV challenge at 4 weeks of age and are referred to as "pups" or "offspring."

Experimental design. Female cotton rats were bled and then primed by RSV A/Long intranasal infection using a dose of 10^5 PFU/animal in 50 μl. After 56 days (8 weeks), females were set up in breeding pairs with RSV-negative males. The females were bled for serum collection at days 63, 84 (just before delivery), and 195 postpriming. At days 63, 70, and 77, different groups of pregnant cotton rats were immunized with UC-2 F VLPs or UC-3 F VLPs with 100 μg total VLP protein/animal (20 μg F protein) or with TNE buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA) (Fig. 1). At days 70 and 77, different groups of pregnant cotton rats were immunized with DS-Cav1 F VLPs or post-F VLPs with 100 μg total VLP protein/animal (20 μg F protein) (Fig. 1). Dams delivered pups at approximately day 84. All pups were eye bled and challenged with RSV A/Long (10^5 PFU/animal) at 4 weeks of age. On day 4 postinfection, all pups were sacrificed for determination of nose and lung tissue viral titers, lung histopathology, and expression of mRNA corresponding to Th1 (IFN-γ) and Th2 (IL-4, IL-6) cytokines, as previously described (15). An additional group of five females remained unprimed and unvaccinated to serve as a source of pups that remained uninfected and provided basal levels of lung gene expression for comparison. Dams were kept in the study for an additional 111 days after delivery of the pups for additional serum collection. The results were collected from two independent experiments (experiments XV-176 and XV-194).

Preparation of RSV, RSV plaque assays, and antibody neutralization. RSV was propagated in HEp-2 cells (ATCC CCL-23), and RSV plaque assays were accomplished on HEp-2 cells as previously described (15, 33) using lung and nose tissue homogenates from two independent experiments. Antibody neutralization titers were determined as previously described (15, 33) in a plaque reduction assay and defined as the reciprocal of the dilution of serum that reduced the virus titer by 60%. The results from two independent experiments were also combined.

Lung histopathology. Lungs were dissected and inflated with 10% neutral buffered formalin to their normal volume and then immersed in the same fixative solution. After fixation, the lungs were embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E). An average pathology score was determined for each group based on four parameters of pulmonary inflammation: peribronchiolitis, perivasculitis, interstitial pneumonia, and alveolitis. The slides were scored blindly on a severity scale of from 0 to 4 as previously described (15). The results from two independent experiments were combined.

Cytokine gene expression by real-time PCR. Total RNA was extracted from homogenized lung tissue using an RNeasy purification kit (Qiagen). One microgram of total RNA was used to prepare cDNA in a volume of 20 μl (QuantiTect reverse transcription kit from Qiagen). cDNA was diluted to 10 μg/ml, and 3 μl was used for each 25-μl reaction mixture for real-time PCR (QuantiFast SYBR green PCR kit; Qiagen) with final primer concentrations of 0.5 μM. The primer sequences for β-actin, IFN-γ, and IL-6

were those described previously (15). Reactions were set up in 96-well plates, and amplifications were performed on a Bio-Rad iCycler apparatus (MyiQ Single Color). The delta threshold cycle (C_T) method was used to calculate the relative levels of gene expression, which were normalized to the level of expression of β-actin as a housekeeping gene. The results shown are from randomly selected pups from each litter obtained in experiment XV-194. The results are representative of those from two experiments.

ELISA protocols. For determination of anti-F protein or anti-G protein serum antibody titers in serum samples, the wells of microtiter plates (Thermo Fisher/Costar) were coated with either purified soluble prefusion (DS-Cav1) F protein or soluble postfusion F protein and the plates incubated for 24 h at 4°C. The wells were then incubated with phosphate-buffered saline (PBS)–2% bovine serum albumin (BSA) for 16 h. Different dilutions of sera in 0.05% Tween and 2% BSA were added to each well, and the plates were incubated for 2 h at room temperature. After six washes in PBS, sheep anti-mouse immunoglobulin antibody coupled to horseradish peroxidase (HRP; catalog number A5906; Sigma) was added in 50 μl PBS–2% BSA, and the plates were incubated for 1.5 h at room temperature. Bound HRP was detected by adding 50 μl 3,3′,5,5′-tetramethylbenzidine (TMB; catalog number 34028; Thermo Fisher) and incubating for 5 to 20 min at room temperature until a blue color developed. The reaction was stopped with 50 μl 2 N sulfuric acid. The color was read in SpectraMax Plus plate reader (Molecular Devices) using SoftMax Pro software. The amounts of IgG (in nanograms per milliliter) bound to the wells were calculated using a standard curve generated using defined amounts of purified IgG.

Statistical analysis. Comparisons of the various groups were accomplished by Student *t* tests or analysis of variance (ANOVA) followed by the Tukey *post hoc* test or the Kruskal-Wallis nonparametric test as indicated in the figure legends.

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